"Production and Characterization of Bioactive Compounds of Microbial Origin against Multi Drug Resistant strains of *Escherichia coli*"

A Synopsis submitted to

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For approval from

RRC Microbiology, S.R.T.M University, Nanded

For

Registration to Doctor of Philosophy in Microbiology

Under the Faculty of Science and Technology

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March 2022

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Abstract:

Escherichia coli is a common inhabitant of gastrointestinal tract of humans and animals; however there are several pathogenic strains of *E.coli* which causes infections viz. diarrhoeal diseases, urinary tract infections, sepsis or meningitis, etc. Cephalosporin, ampicillin and fluoroquinolones are drugs used to treat infections caused by *E.coli. E.coli* shows resistance against these drugs by developing efflux pumps, target site mutation and loss of outer membrane porin. The increased resistance to antibiotics has failed to treat infection and it leads in increasing mortality rate among hospitalized patient. The bacterial and fungal spp. present in unique ecological niche has regulatory gene for bioactive compound production which expresses only in stress condition. The producer organism has survival and growth benefit by suppressing the growth of competeting organism. These compounds might have ability to inhibit the growth of MDR *E.coli.* In present investigation, MIC of antibiotics (viz. ceftriazone, ciprofloxacin, carbapenems, amoxicillin, ampicillin, and fluoroquinolones) against MDR *E.coli* will be determined. Novel bioactive compound producing bacteria and fungi will be isolated from unique ecological niche. The screening and production of bioactive compound will be carrying out and purified by fractional purification method. The potent bioactive compound will be analyzed structurally and their bioactivities against MDR strains of *E.coli* will be evaluated. The MDR strains of *E.coli* will be obtained from clinical sample and culture collection centre, the MIC of antibiotics (viz. ceftriazone, ciprofloxacin, carbapenems, amoxicillin, ampicillin, fluoroquinolones will be determined). The novel bioactive compound will be obtained either from bacterial and fungal isolates which can be effectively used for the control of the growth of MDR strains of *E.coli* responsible for causing diarrhoeal diseases, urinary tract infections, sepsis or meningitis, etc. The lead will be best candidate for development of drug against MDR strains of *E.coli.*

Keywords: *E.coli*, MDR, bioactive compound, MIC

Introduction

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus Escherichia, commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious disease. The harmless strains are part of the normal microbiota of the gut, and can benefit their hosts by producing vitamin K12 [1] and preventing colonization of the intestine with pathogenic bacteria, having a mutualistic relationship [2][3]. Specific pathotypes of *E.coli* are capable of causing disease in healthy individual's viz. enteric/diarrhoeal disease, urinary tract infections (UTIs) and sepsis/meningitis (4). The bacterial pathogen most commonly associated with endemic forms of childhood diarrhea is *Escherichia coli* (5). The six categories of diarrheagenic *E. coli* have been described: entero-pathogenic *E. coli* (EPEC), entero-toxigenic *E. coli* (ETEC), enteroinvasive E. coli (EIEC), entero-hemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (6). Two additional categories- cell-detaching *E. coli* (CDEC) (7) and cytolethal distending toxin-producing *E. coli* (CLDTEC) (8). The entero-hemorrhagic *E. coli* (EHEC) strains are the most publically recognized strains and include *E. coli* O157:H7 as well as Shiga-toxin producing *E.coli*. These strains have a very low infectious dose and are transmitted through infected food especially meat and unpasteurized beverages. Diarrhea is a leading cause of morbidity and mortality among children in developing countries. (9)

Pathogenic *E. coli* strains use a multi-step scheme of pathogenesis that is similar to that used by other mucosal pathogens, which consists of colonization of a mucosal site, evasion of host defences, multiplication and host damage. Most of the pathogenic *E. coli* strains remain extracellular, but EIEC is a true intracellular pathogen that is capable of invading and replicating within epithelial cells and macrophages (10). Urinary tract infection (UTI) can be caused by Gram-negative bacteria such as *Escherichia coli, Klebsiella species*. *E. coli* is the most common organism causing both community as well as hospital acquired UTI. (11) Pathogenic *E. coli* strains possess specific adherence factors that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra. Most frequently these adhesins form distinct morphological structures called fimbriae (also called pili), which can belong to one of several different classes. The Afa adhesins that are produced by many diarrheagenic and

uropathogenic *E. coli* (UPEC) are described as a fimbrial adhesins, but in fact seem to have a fine fibrillar structure that is difficult to visualize. Adhesins of pathogenic *E. coli* can also include outer-membrane proteins, such as intimin of UPEC and EHEC, or other non-fimbrial proteins. Some surface structures trigger signal transduction pathways or cytoskeletal rearrangements that can lead to disease. The trigger signal transduction pathways are secreted toxins and other effector proteins that affect an astonishing variety of fundamental eukaryotic processes. Concentrations of important intracellular messengers, such as cyclic AMP, cyclic GMP and Ca2+, can be increased, which leads to ion secretion by the actions of the heat-labile enterotoxin (LT), heat-stable enterotoxin a (STa) and heat-stable enterotoxin b (STb), respectively — all of which are produced by different strains of ETEC. The Shiga toxin (Stx) of EHEC cleaves ribosomal RNA thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells.[12] A high percentage of the isolated *E. coli* strains were multidrug resistant (MDR) to penicillins: ampicillin, amoxicillin, and amoxicillin-clavulanic acid; cephalosporins: ceftazidime and cefotaxime; carbapenems: meropenem and imipenem, and are harboring the blaTEM, blaCTX, and blaKPC gene. [5] The rise of resistance poses a threat to all nations. Enzyme synthesis, efflux pumps, and porin mutations are the main methods by which Enterobacteriaceae acquire resistance [13].

In the last few years, emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no effective antimicrobial agents available for infections caused by these bacteria. According to the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), multi-drug resistant (MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories. [5] MDR bacteria are the principal cause of failure in the treatment of infectious diseases, resulting in increases in the term and magnitude of morbidity, higher rates of mortality, and a greater health cost burden.[14,15] However, recently researchers have been investigated the activity of non-antimicrobial agents such as pro-anthocyanidin to prevent MDR bacterial infections. Multi-drug resistant *E. coli* are widely distributed in hospitals and are increasingly being isolated from community. Thus, it is urgent need to find out new antimicrobial agents. ([15, 16 and 17] However,

new families of antimicrobial agents have a short life expectancy.[16] Several monitoring programmes have been initiated to generate baseline data about the prevalence of MDR in different bacterial species, including *E. coli*[17, 18] many studies from Europe [19] and USA (20,21) have investigated MDR among *E. coli* isolates. Most bacterial isolates from Asian and African countries have shown high MDR rates. [22]

Since ancient times, mankind has been exploring nature for bioactive compounds to treat different ailments. More than 100000 natural products have been identified in the last 150 years, which include highly assorted chemical classes such as polyketides, alkaloids, non-ribosomal peptides, isoprenoids or phenyl-propanoids. [23, 24] Among the prospective sources of natural products, bacteria are prolific sources and a vast majority of these products are produced by "Actinobacteria". [25,26, and 27] Hence bacteria along with fungi from unique ecological niche will be isolated and screened against MDR strain of *E. coli.* The bacterial and fungal isolates showing anti *E. coli* activity will be identified by ribosomal r-RNA sequencing and used for production of bioactive compounds. The bioactive compounds will be purified by using fractional purification methods. The purified bioactive compound again used for bioactivity against MDR strain and characterized for molecular structure. The interaction of purified bioactive compounds with target site will be analyzed.

Review of Literature:

Escherichia coli is a common pathogen linked with community-associated as well as nosocomial infections. In the last few years, the emergence and wide dissemination of *E. coli* strains showing resistance to broad-spectrum of antimicrobial agents has been reported. Multi-drug resistance is increasing day by day and becoming a global health care issue. Hence, there is an urgent need to combat the multidrug resistance uropathogens, so the discovery of novel, efficient, and safe antibiotics from unexplored ecological niches is needed. Actinobacteria have great eminence of their potential for producing bioactive compounds. The aim of the present study is production and characterization of bioactive compounds of microorganism origin against MDR strains of Escherichia coli.

Tanabe et.al reported that pathogenic *E. coli* are divided into two major groups: diarrheagenic (DEC) and extraintestinal pathogenic *E. coli* (ExPEC), which causes diarrhea and extraintestinal infections, such as neonatal meningitis, sepsis, and urinary tract infections (UTIs), respectively. [(28)] Sibero et.al, mentioned that *Escherichia coli* is one of the common bacteria which infects humans. These bacteria cause urinary tract infection, nosocomial bloodstream infection, meningitis, bacteraemia, and gastrointestinal disease. [(29)]

Tilahun et al reported that the Carbapenems are the medications of choice for extended-spectrum beta-lactamase and AmpC producers, but alternatives are currently needed because carbapenem resistance is increasing globally. Enzyme synthesis, efflux pumps, and porin mutations are the main methods by which Enterobacteriaceae acquire resistance to carbapenems [(13)]. Hudault S et.al revealed that the wild-type *E. coli* and *K. pneumoniae* clinical isolates are susceptible to quinolones, but resistance to these agents in Gram-negative bacteria has increased in recent years, probably caused by excessive and inappropriate use of these drugs. Particularly, due to under-dosing and mono-therapy against moderately susceptible pathogens, FQ resistance has developed among common pathogens, like *E. coli and Klebsiella spp.,* mainly conferred by ESBLs and AmpC enzymes [[2]]. Reid G et.al, mentioned that the antimicrobial drug resistance in commensal *E. coli* in a large population of preschool-age children from urban settings of low-resource countries showed a high prevalence (70%–95%) of healthy carriers of *E. coli* resistant to a number of older antimicrobial agents viz. ampicillin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, and streptomycin [(3)].

Castillo et.al, reported that the multidrug-resistant bacteria, antibiotic resistance profile of *E. coli* isolated from community and hospital-acquired urinary tract infections shows resistance toward ciprofloxacin (47.3%), levofloxacin (43.6%) and cephalosporins (27.6%) was observed [(30)]. Sharma et.al, *E. coli* was isolated from North Indian patients with UTI. *E coli* showed absolute resistance (100 %) to cotrimoxazole and tetracycline. E. coli showed high amount of resistance to fluoroquinolones (75 %) and aminoglycosides (67 %) [(31)].

Devanand et.al revealed that there is a high prevalence of multidrug resistant E. coli in UTI. Isolates were taken from UTI patients and their susceptibility pattern was studied and

Minimum inhibitory concentration (MIC) was determined by agar dilution method in Mueller Hinton agar [32]. Dan et.al, reported that the sputum, blood and/or cerebrospinal fluid (CSF) samples were taken and *E. coli* strains were isolated from inpatient neonates diagnosed with pneumonia, sepsis and/or meningitis. The minimal inhibitory concentration (MIC) values of the antimicrobial agents selected for this study were determined by an agar dilution technique on Mueller-Hinton agar [33]

Khadayat et.al, reported that the soil samples were collected from different habitats of Nepal. *Streptomyces* species were isolated on ISP4 media, and characterization was performed according to morphological similarity and 16S rRNA sequence similarity using bioinformatics tools. Ethyl acetate extracts of *Streptomyces* species were prepared, and the antimicrobial activity was carried out using agar well diffusion technique. Isolates have shown antibacterial activity against extended-spectrum beta-lactamase- (ESBL-) producing *Escherichia coli* [34]. Daigham et.al, mentioned that the soil samples were collected from gardens located in Nasr City, Cairo, Egypt for the isolation of actinomycetes on starch casein agar (SCA) plates. The incubation of all plates was carried out at 30±2°C for 7 days. The resulting actinomycetes colonies were picked up, streaked, recultivated a number of times on SCA plates, and then incubated at 30°C for 7 days to check its purity. The pure actinomycetes isolates were maintained on SCA slants at 4°^{C} [35]. Ashok kumar et.al, the soil samples were collected; strains of actinomycetes were isolated and subjected to primary screening by giant colony method against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and Aeromonas hydrophilia.* The antibacterial activity was evaluated by well plate method. The zone of inhibition was higher with Actinomycetes AK1 inoculated plates. The antibiotic production with Shake flask fermentation was carried out with Antibiotic production medium. The flasks were shaken at 28℃ and 160 rpm for 5-7 days on the rotator shaker and finally the filtrate was used to test the antimicrobial activity [36].

Zothanpuia et.al, revealed the presence of bioactive compounds in strain DST103 was identified as *Streptomyces cyaneofuscatus* (KY287599) using 16SrRNA gene sequencing, which may be a promising resource for the discovery of novel bioactive metabolites against

wide range of pathogens [37]. R.Vijayakumar et.al, mentioned that actinomycetes were isolated from marine soil sample on starch–casein agar (SCA) plates. These isolates have antimicrobial activity, the antimicrobial compound was extracted from the isolate using various solvents and the antimicrobial efficacies were tested against bacterial and fungal pathogens. The selected isolates were produced and filtered, the filtrate was transferred aseptically into the conical flasks and an equal volume of five different solvents (alcohol, chloroform, distilled water, ethyl-acetate and methanol) was separately added to the cell-free culture filtrates and shaken for 2 hr and the extracted filtrates were tested for their antimicrobial activity by using well-diffusion method [38].

Shetty et.al revealed that a highly potent secondary metabolite producing actinomycetes strain is isolated from marine soil sediments of Visakhapatnam sea coast, Bay of Bengal. Among the actinomycetes strains the broad spectrum strain RSPSN2 was selected for molecular characterization, antibiotic production and its purification. The active metabolite was extracted using ethyl acetate (1:3, v/v) at pH 7.0. The separation of active ingredient and its purification was performed by using both thin layer chromatography (TLC) and column chromatography (CC) techniques [39]. Mohseni et.al, reported that the Samples were collected from the sediments of Caspian Sea. Actinomycetes were isolated and preliminary screening was done using cross-streak method against 2 gram-positive and 4 gram-negative pathogen bacteria. The most potent strains MN2, MN3, MN38, MN39, MN40, MN41, and MN44 were used to extract the antibacterial substances [40]. Mahdiyah et.al, reported that the peat soil samples were collected from the peat soil area of Ban-jarmasin district in South Kalimantan, Indonesia. This study reported that isolates from Indonesian peat which were potentials sources of new antimicrobials [41].

Objectives of Research:

- ✓ Procuring or Isolation of MDR *E. coli* strains from clinical sample
- ✓ Determination of MIC of antibiotics
- ✓ Isolation of bioactive compound producing microorganism from unique sites
- ✓ Screening of bioactive molecules against MDR strain of *E. coli*
- ✓ Production of bioactive compounds
- ✓ Extraction and purification of bioactive compounds
- ✓ Bioassay of purified bioactive compound against MDR strains
- ✓ Characterization of potential bioactive compound
- ✓ Identification of target site in MDR strains for bioactive molecules.

Hypothesis

The bacterial and fungal species from ecological niche have ability to produce bioactive compound. The bioactive compound of microorganisms might have potential to inhibit the growth of MDR *E.coli.* This bioactive molecule will be effective for controlling the disease caused by *E.coli* or augmenting antimicrobial activity of antibiotics effective against MDR *E.coli.*

Methodology to be adopted

- ✓ The MDR strains of *E. coli* will be procured from culture collection Centre and also isolated from clinical sample
- ✓ The MIC of antibiotics will be determined against procured *E. coli* & strains of *E. coli* isolated from clinical sample
- ✓ Soil/water sample will be collected from unique ecological niche for isolation of bacterial and fungal species which have ability to produce bioactive compound against MDR strain of *E.coli*
- ✓ The isolated microbial isolates will be identified on the basis of morphological and biochemical characteristics and will be used for production of bioactive compounds

- ✓ The bioactive compound from production medium will be used for bioactivity study against MDR strain of *E. coli.*
- ✓ The microbial isolates showing potential of bioactive compound production will be identified by using 16S /18S rRNA sequencing.
- ✓ The stress induction study will be carried out for production of novel bioactive compound from bacterial and fungal isolates.
- ✓ The production broth will be used for fractional purification of bioactive compound and for its structural analysis.
- ✓ The interaction of bioactive molecules with target site in MDR *E. coli* will be investigated.

National Status:

Archana Singh and Padma Singh reported that Soil samples were collected from the Ganga riverbed, wheat and rice fields, Uttarakhand, India. Some antimicrobial producing actinobacteria strains isolated from the soil and cross streaked against multidrug resistant uropathogens. Isolates showed good antimicrobial activity against *Pseudomonas aeruginosa, Enterobacter sp., Bacillus subtilis,* and *Staphylococcus aureus* uropathogens. Among them, ACG1 showed a broad range of activity against all the tested uropathogens. ACG1 was identified based on morphological, cultural, physiological, and biochemical properties, as well as the 16S ribosomal ribonucleic acid sequence, and was assigned the name *S. atacamensis* [(42)].

Singh et.al, Central Drug Research Institute, Lucknow, India reported that the antimicrobial potential of novel actinomycetes has been evaluated by initial screening of six soil samples. Primary and secondary screening was performed against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Candida albicans, Candida tropicalis, Trichophyton rubrum,* and other MDR bacterial and fungal test strains. Five most active microbial strains were identified using 16S rRNA sequence homology and designated as *Streptomyces xanthophaeus* MTCC 11938, *Streptomyces variabilis* MTCC 12266, *Streptomyces xanthochromogenes* MTCC 11937, *Streptomyces levis* EU 124569, and *Streptomyces sp*. NCIM 5500. Four antibacterial and three antifungal compounds isolated from the above five isolates were purified and partially

characterized using UV absorption and IR spectra. Two antibacterial metabolites, belong to chromone and peptide antibiotic, respectively. The antifungal compounds were found to be of non-polyene nature [(43)].

Aparna V et.al, SRM University, Kattankulathur, Tamilnadu, India reported that *Pseudomonas aeruginosa* and *Escherichia coli* are resistant to wide range of antibiotics rendering the treatment of infections very difficult. A main mechanism attributed to the resistance is the function of efflux pumps. MexAB-OprM and AcrAB-TolC are the tripartite efflux pump assemblies, responsible for multidrug resistance in *P. aeruginosa* and *E. coli* respectively. This study reported that, lanatoside C and diadzein could be promising efflux pump inhibitors and effective to use in combination therapy against drug resistant strains of P. aeruginosa and E. coli [(44)]. Muthukrishnana et.al, P.S.G. College of Arts and Science, Coimbatore, Tamil Nadu, India reported that the soil samples were collected for isolation of actinomycetes. The prospective strain MK MSt033 was determined as *Nocardiopsis sp.* The strain displayed effective antimicrobial activities against both bacterial and fungal pathogens such as *Escherichia coli, Staphylococcus aureus, Penicillium sp.,* and *Aspergillus flavus* respectively. The identified isolate *Nocardiopsis sp.* MK MSt033 exhibited potential antimicrobial activities against selected microbial pathogens. Thus, the soil inhabiting *Nocardiopsis sp.* has explored for pharmaceutically active compounds with promising medical applications [(45)].

Khadayat et.al, reported that the *Streptomyces* species were isolated from Nepalese soil on ISP4 media. Ethyl acetate extracts of *Streptomyces* species were prepared, and the antimicrobial activity was carried out using agar well diffusion technique against extended-spectrum beta-lactamase- (ESBL-) producing *Escherichia coli.* The *Streptomyces* species-derived ethyl acetate extracts from Nepalese soil demonstrate potential activity against ESBL-producing *E. coli.* Thus, they are potential candidates for antibiotics manufacturing in the future. [(46)]

International status:

Apsari PP et.al, Department of Biology, West Java, Indonesia reported that this study aims to look for antibacterial compounds from rhizosphere actinomycetes against bacteria in urine of UTI patients *(Escherichia coli, Citrobacter braakii, Acinetobacter calcoaceticus,* and *Klebsiella*

pneumoniae). The screening of actinomycetes was conducted based on the paper disc method. Potential actinomycetes were identified by 16S rRNA gene to reveal the species. The screening results showed that ARJ 16, 24, and 36 had a wider inhibition zone than others. The highest abundance of bioactive compound of crude extract was found in Propane, 1, 2-dichloro, n-Hexadecanoic acid and Carbonochloridic acid, 2-chloroethyl ester, respectively. Identification of potential actinomycetes based on 16S rRNA gene showed that ARJ 16 and ARJ 24 were highly similar to Streptomyces sp. In 99% and ARJ 36 was similar to Streptomyces tendae in 98% [(47)].

Norouzi et al., Department of Biology, University of Isfahan, Iran reported that marine actinomycetes isolated from the Caspian Sea, Iran. Primary screening via cross-streak method revealed that 3 strains: MN2, MN39, and MN40 produce antimicrobial agents with wide spectrum activity. The potent strains were characterized morphologically, and then identified genetically using 16S rRNA analysis showed that the potent strains belong to the genus Streptomyces. After that, the bioactivity of the ethyl acetate extracts of liquid culture against some MDR bacteria has been studied using disc diffusion method. The results of disk diffusion method indicated that the potent isolates, MN39 and MN2 produce biomolecules with antibacterial activity against MDR bacteria especially methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE) [(48)]. Ryandini et al. Faculty of Biology, Jenderal Soedirman University, Indonesia revealed that Streptomyces sp. SA32 isolated from mangrove rhizosphere mud was selected to inhibit the growth of MDR bacteria *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Enterobacter cloaceae,* and *Enterococcus sp. E. coli* was sensitive to crude extracts of Streptomyces sp. SA32. The bioactive compounds analyzed using thin layer chromatography and phytochemical methods showed that the spot with RF 0.63 and 0.68 was polyketides compound and that with RF 0.74 was flavonoid compound. The bioautography assay on the TLC plate confirmed the absence of MDR bacterial growth on polyketides and flavonoid spots. The synthesis of antibacterial compounds was also confirmed by the successful analysis on both non-ribosomal peptide synthetase (NRPS) and polyketides synthase (PKS) gene sequences [(49)].

Zhang et.al, College of Animal Science and Technology, Guangxi University, Nanning, China evaluated the antibacterial mechanisms of phenolic acids as natural approaches against multi-drug resistant *Escherichia coli.* Compound phenolic acid improves the sensitivity of *E. coli* to antibiotics (ceftriazone sodium, amoxicillin, fosfomycin, sulfamonomethoxine, gatifloxacin, lincomycin, florfenicol, cefotaxime sodium, and rifampicin), causes the flagellum to fall off, breaks the structure of the cell wall and cell membrane, and leads to macromolecules leaks from the cell. This evidence elaborated the potential therapeutic efficacy of CPA 19 and provided a significant contribution to the discovery of antibacterial agents [(50)]. Sibero et.al, Department of Coastal Resources Management, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semaran, Indonesia reported that marine sponge associated fungi have potential as source of new compound to combat MDR *E. coli.* Marine sponge-associated fungi isolated from Panjang Island, Central of Java, Indonesia, to screen potential fungi against MDR *E. coli.* Sponge-associated fungi screened, there were 7 isolates showed antibacterial activity against MDR *E. coli.* The best inhibition zone produced by MPS 14.1/MT 02 and MPS 14.3/MT 04 from sponge PP.SP.16.14. According to fungi identification result fungus MPS 14.1/MT 02 was identified as *Trichoderma asperellum* while MPS 14.3/MT 04 was identified as *Trichoderma reesei.* Sponge identification leaded the PP.SP.16.14 as *Cinachyrella sp.* [(51)]

Significance of study:

The novel new bioactive compound will be isolated from bacterial and fungal isolates isolated from unique ecological niche. The novel compound may be free from side effects or minimum side effects. The effective concentration will be less to achieve maximum efficacy and it may also augments the activity of drugs used for treating infection caused by MDR E.coli.

Proposed work Plan:

Year-wise Plan of work and targets to be achieved:

Year	Target
V 0-1 Year	✓ Course work completed ✓ References collection & review writing
1-2 year	✓ Isolation, screening and production bioactive compounds ✓ Purification and characterization of Bioactive compounds
2-3 Year	✓ The interaction of bioactive molecules with target site in E. coli ✓ Resulting and data analysis
3-4 Year	✓ Thesis writing and submission to University.

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